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# Complementary DNA sequence encoding the major neural cell adhesion molecule isoform in a human small cell lung cancer cell line

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**Abstract**

The neural cell adhesion molecule (N-CAM), a member of the immunoglobulin gene superfamily mediating homophilic cell-cell adhesion in a neuroendocrine system, is preferentially expressed in human small cell lung cancer (SCLC). Immunoprecipitation of a panel of SCLC cell lines by monoclonal antibodies (mAbs) specific for N-CAM detects mainly the 145-kDa isoform. This result was correlated with Northern blotting where a single 6.2-kb mRNA was detected in nine SCLC cell lines. To determine cDNA sequence encoding the N-CAM isoform, we selected several cDNA clones encoding N-CAM isolated from OS2-R, a SCLC cell line established in our laboratory. Based on the analysis of the full-length cDNA obtained from two clones, the sequence of this 145-kDa isoform was shown to be essentially identical to that of the 140-kDa N-CAM isoform of neuroblastoma except for a single base pair changed at position 1620 without changing amino acid encoded.

*Key words:* Neural cell adhesion molecule; N-CAM; Small cell lung cancer; Complete sequence

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*Abbreviations:* N-CAM, neural cell adhesion molecule; SCLC, small cell lung cancer; mAb, monoclonal antibody; cDNA, complementary DNA; NSCLC, non-small cell lung cancer; GPI, glycosylphosphatidyl inositol; VASE, variable alternatively spliced exon; MSD1, muscle specific domain 1; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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## 1. Introduction

Small cell lung cancer is a highly aggressive malignant neoplasm, which exhibits a rapid growth rate, and frequently invades and metastasizes [1]. The high rate of tumor recurrence and the frequent presence of subclinical metastases may preclude the possibility of surgical cure even at early stages of SCLC. Therefore, it is important to distinguish SCLC from non-small cell lung cancer (NSCLC) for the selection of therapeutic arms for lung cancer patients.

In immunoperoxidase staining using monoclonal antibodies (mAbs) reacting with SCLC, a number of anti-SCLC mAbs have been shown to recognize an antigen expressed preferentially on SCLC among lung cancers [2,3]. Further immunohistochemical and molecular analyses have revealed that the antigen is a 120- to 165-kDa glycoprotein expressed in the neuroendocrine organs, called neural cell adhesion molecule (N-CAM) [4].

N-CAM, a member of immunoglobulin gene superfamily, is a cell surface sialoglycoprotein mediating homophilic cell-cell adhesion in a variety of cell types in the embryo and in neurons, neuroendocrine cells, glia, and the neuromuscular junction in adults [5–7]. This molecule is highly conserved in structure and function during evolution [6], and a series of structurally distinct isoforms are expressed in a developmentally regulated and tissue-specific manner [8]. Diversity of the N-CAM isoforms in man is derived by alternative mRNA splicing from a single gene [9], which is on chromosome 11 q23.1 [10], and also by post-translational modifications including phosphorylation, sulfation [11] and glycosylation, in particular, polysialylation [12]. In brain, three membrane associated forms have been well characterized. Two large isoforms of 180 and 140 kDa are the transmembrane glycoproteins with differing length of cytoplasmic region, and the 120-kDa isoform is one with linking into the membrane via a glycosylphosphatidyl inositol (GPI) anchor [13]. N-CAM isoforms expressed in human skeletal muscle have been identified as a 140-kDa transmembrane protein, two GPI-linked isoforms (125 kDa and 155 kDa), and a 115-kDa secretory protein [14,15]. N-CAM is also expressed in human natural killer cells, and has been shown to be essentially identical to the predominant 140-kDa isoform present in neuroblastoma, but distinct from the structure in muscle tissues [16].

The coding region of N-CAM gene consists of at least 19 exons [5]. Exons 1–14 encode the extracellular domain which is a commom portion of all N-CAM isoforms. Exons 15 and 16 encode GPI-linkage to the cell surface membrane and the transmembrane domain, respectively. The remaining exons encode the cytoplasmic domain. Depending on the choice of 3′-end alternatively spliced exons (15–19), three representative isoforms of N-CAM polypeptide may be expressed in brain. Alternative splicing has been observed in two other regions of the *N-CAM* gene. A 30-bp alternatively spliced exon termed VASE between exons 7 and 8 was detected not only rodent's nervous system and heart [17,18] but also in human neuroblastoma [19], human adult brain and SCLC cell lines [20]. A 108-bp sequence termed MSD1 was found between exons 12 and 13 in human skeletal muscle cDNA [14]. This exon consists of four small exons, MSD1a, MSD1b, MSD1c and triplet AAG. Another exon, SEC, that presumably generates a secret form of N-CAM is also inserted

between exons 12 and 13 [15]. These findings indicate that the splicing patterns in these different regions of the gene is much more complex than first assumed.

Carbone et al. have demonstrated that N-CAM is expressed in a small population of NSCLC which is positive for neuroendocrine markers including L-dopa decarboxylase, dense core secretory granules, synaptophysin and chromogranin A [21]. They have speculated that N-CAM may play a functional role in the neuroendocrine differentiation of lung cancer cells regardless of their histological types. However, the primary structure of lung cancer-derived N-CAM has not been reported.

For understanding more precisely the derivation and the development of SCLC and NSCLC with neuroendocrine phenotypes, it is at first important to characterize and identify the primary structure of N-CAM expressed in these tumors, although it is also efficient to study well known alternatively spliced regions such as VASE, MSD1, SEC and exon 15–19 using PCR or Northern blotting analysis with isoform specific oligonucleotide probes [20–22]. In this study, we determined the nucleotide and the predicted amino acid sequence of the full length N-CAM-coding region for a 145-kDa form isolated from a cultured SCLC cell line.

## 2. Materials and methods

### 2.1. Cell lines

The SCLC cell lines OS1, OS2, OS2-R, OS2-RA, OS3, OS5 and OS7 were established in our laboratory [23,24]. SBC1 was obtained from Japanese Cancer Research Resources Bank. The other SCLC cell line H69 and a large cell lung cancer cell line, Lu99, were the kind gift of Dr Y, Shimosato, National Cancer Center Research Institute, Tokyo, Japan. The PC9 lung adenocarcinoma cell line was a generous gift of Dr Y, Hayata, Tokyo Medical College, Tokyo, Japan. The cell lines of neuroblastoma, 1MR32 and NB1, were obtained from the American Type Culture Collection, Rockville, MD. Lu99 and PC9 were serially passaged in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. This medium was designated complete medium. All SCLC and neuroblastoma cell lines were maintained in complete medium supplemented with 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM 17β-estradiol and 30 nM sodium selenite.

### 2.2. Antibodies

Anti-SCLC mAbs, ITK-2 and ITK-3 were generated in our laboratory [25], and MOC-1 [26] and NKH-1 [27] were purchased from Bio-Science Products, Emmenbrücke, Switzerland, and Coulter Immunology, Hialeah, FL, respectively. 11 G1, an IgG1 mAb against a murine hepatoma [28], was used as a control.

### 2.3. Immunoprecipitation

Immunoprecipitations with mAbs or control mAb were performed by the method of Kubo et al. [29]. Briefly, cells were radiolabeled with $Na^{125}I$ by the iodogen met-

hod, and subsequently lysed in 10 mM Tris/HCl (pH 7.4) containing 150 mM NaCl, 0.5% Triton X-100, 0.2 mM phenylmethyl sulfonyl fluoride and 50 units/ml aprotinin at 4°C for 15 min. Nuclei and cell debris were removed by centrifugation at 10 000 × *g* for 10 min. Cell lysates were immunoprecipitated with rabbit anti-(mouse-Ig)-coated protein-A-Sepharose (Sigma Chemical Co., St. Louis, MO) conjugated with each mAb. After extensive washing, immunoprecipitated materials were dissolved in 62.5 mM Tris/HCl (pH 6.8) containing 10% glycerol, 2.6% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol and 0.025% bromophenol blue, and boiled for 5 min. The samples were electrophoresed on a 10% polyacrylamide gel according to the method of Laemmli [30]. The gels were dried and exposed to Kodak X-Omat AR films at −70°C.

### *2.4. cDNA probe preparation*

Total RNA was isolated from a SCLC cell line, OS1, as described by Chomczynski et al. [31]. Poly(A)$^+$ RNA was isolated by using Oligotex-dT30 as recommended by the manufacturer (TaKaRa Shuzo Co, Ltd, Kyoto, Japan). cDNA was synthesized with a cDNA Synthesis System Plus kit according to the manufacturer's instructions (Amersham, UK). To generate a human N-CAM cDNA probe by PCR, we prepared a pair of oligonucleotide primers based on the nucleotide sequence of the 125-kDa isoform of human skeletal muscle N-CAM [32], using Pharmacia Gene Assembler Plus DNA synthesizer (Pharmacia LKB Biotechnology, Tokyo, Japan). The sense primer was 5′CCGCGAATTCCTGCAGGTGGATATTGTTCCCA-3′, and the antisense primer was 5′TTGCACTTCTAGAAAGTCTTCCTTAAGCGCC-3′. The 5′ end of the sense primer and the 3′ end of the antisense primer contain *Eco*RI sites for cloning. PCR reactions were performed (25 cycles at 94°C, 30 s; 55°C, 1 min; 72°C, 1 min) using a Gene Amp Kit (Perkin-Elmer Cetus, Norwalk, CT). PCR products were digested with *Eco*RI, subcloned into pBruescript (Stratagene, La Jolla, CA), and were sequenced with a DNA sequencing kit according to the manufacture's protocol (Sequenase ver. 2.0, United States Biochemical Corporation, Cleveland, OH). The result was compared with previously published sequences of human N-CAM isoform from brain and muscle [19,32]. This probe was designated N294.

### *2.5. Northern blotting*

Total RNA and poly(A)$^+$ RNA were prepared as described above. Twenty micrograms of total RNA or 2 μg of poly(A)$^+$ RNA were separated on 1% formaldehyde agarose gels, and transferred to Hybond-N$^+$ nylon filters as recommended by the manufacturer (Amersham). OD260 and ethidium bromide staining were used to estimate RNA amounts loaded per well. $^{32}$P-labeled probe was generated by random priming (Multiprime DNA labeling system, Amersham). Hybridization was performed by the method of Maniatis et al. [33]. Blots were washed at high stringency, finally, in 0.1 × SSC and 0.1% SDS at 65°C for 15 min, and exposed to Kodak X-Omat AR films at −70°C for an appropriate period.

### 2.6. Construction and screening of a λgt10 cDNA library

Poly(A)$^+$ RNA was prepared from a SCLC cell line, OS2-R, and double strand cDNA was synthesized as described above. λgt10 cDNA library was constructed by a cDNA cloning system λgt10 kit (Amersham). Briefly, after ligation of *Eco*RI adaptors, cDNA over 500 base pairs long was size selected by column chromatography and then inserted into *Eco*RI-digested λgt10 DNA. Approximately $5 \times 10^5$ plaques were screened with $^{32}$P-labeled N-CAM cDNA probe N294, and positive clones were plaque purified by recloning.

### 2.7. DNA subcloning and sequencing

Recombinant DNA from λ phage was prepared by standard methods, and *Eco*RI digested fragments were purified from a preparative agarose gel. These inserts were subcloned into pBluescript and a series of deletion mutants were constructed from the cDNA inserts by the use of exonuclease III and nuclease S1 [34]. Sequencing of the DNA was achieved by the dideoxy chain termination method using [$^{35}$S]dATP and Sequenase as described above.

## 3. Results

### 3.1. Immunoprecipitation

Three well-characterized SCLC cell lines, two neuroblastoma cell lines and PC9 lung adenocaricinoma cell line as control were examined to determine which types of N-CAM isoforms were expressed mainly in SCLC by immunoprecipitation. ITK-2 bound to a 145-kDa protein from OS1, OS3 and H69 SCLC lines, and to a smear-like protein ranging from 140 to 200 kDa from 1MR32 and NB1 neuroblastoma cell lines and also H69 SCLC cell line (Fig. 1A). The smear-like band indicates that the N-CAM expressed in these cell lines contains high level of α2-8 linked polysialic acid [13]. Neither the 145-kDa nor the smear-like band was detected in the immunoprecipitate of PC9. When OS3 cells were used as a target, ITK-2, ITK-3, MOC-1 and NKH-1 also precipitated the positive band at 145 kDa (Fig. 1B). No positive band was seen when 11G1, an IgG1 mAb against an unrelated tumor, was used as the first antibody in this assay. Another weak band seen ahead of the 145-kDa band in OS3 cells (Fig. 1A, lane B; 1B, lanes A and D) seemed to be a non-specific band, because it was weakly but clearly detected in the control experiment using 11G1 mAb on the autoradiogram. (Fig. 1B, lane E).

### 3.2. Northern blotting

To confirm the results shown by the immunoprecipitation, Northern blot analysis was carried out using N294 as an *N-CAM* probe. This probe contains 294 base pairs sequence of 5′ end of 125-kDa human skeletal muscle N-CAM [32] which corresponds to exons 1 and 2 (Fig. 3). It seems possible that this probe can detect alternatively spliced mRNAs present in human SCLC cell lines, because this region has been reported to be the common site in all human N-CAM isoforms [16,19,32]. Nor-

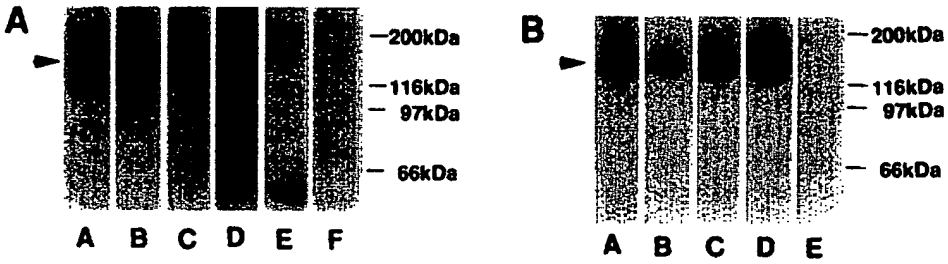


Fig. 1. (A) Immunoprecipitation of extracts of SCLC and neuroblastoma cell lines by ITK-2. $^{125}$I-labeled proteins from Triton X-100 lysates of OS1 (A), OS3 (B), H69 (C), IMR32 (D), NB1 (E) and PC9 (F) were immunoprecipitated by ITK-2 and analysed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. (B) Immunoprecipitation of an extract of OS3 cells by several mAbs. $^{125}$I-labeled protein from Triton X-100 lysates of OS3 cells was immunoprecipitated by ITK-2 (A), ITK-3 (B), MOC-1 (C), NKH-1 (D) and 11 Gl (E), and analysed by SDS-PAGE under reducing conditions. Positions of size markers are indicated on the right of both figures.

thern blot analysis using 20 μg of total RNA showed a single band at approximately 6.2 kb in 9 SCLC cell lines (Fig. 2). In addition to the 6.2-kb band, neuroblastoma cell lines, 1MR32 and NB1, showed a band at 7.4 kb which was also detected in the lane of IMR32-mRNA. The 7.4-kb mRNA has been shown to encode the 180-kDa

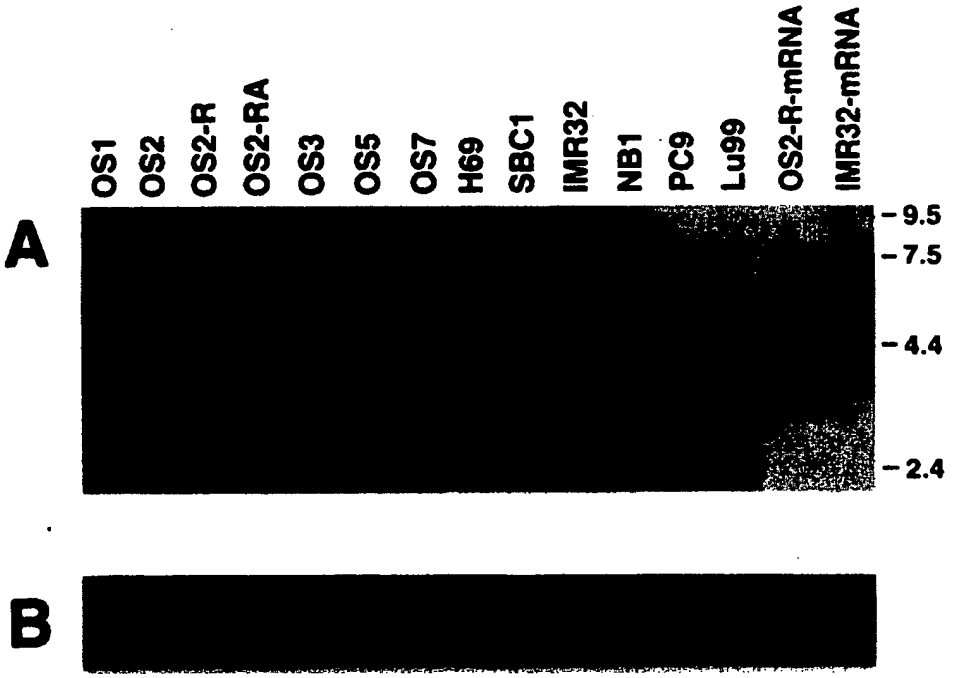


Fig. 2. (A) Northern blot analysis of 20 μg of total RNA or 2 μg of poly(A)$^+$ RNA prepared from SCLC and neuroblastoma cell lines. The membranes were hybridized with radiolabeled N294 probe and washed under high stringency. Positions of size markers are indicated on the right. B, Ethidium bromide staining of 28 S ribosomal RNA showed that almost equal amounts of total RNA had been loaded per well.

isoform of N-CAM expressed in some neuroblastoma cell lines [35]. Neither the 6.2-kb nor the 7.4-kb band was detected in the lane of PC9 or Lu99. A 4-kb band which was not seen in the analysis of poly(A)$^+$ RNA seems to be an artifact, because some non-specific trapping of signal may occur at 4 kb where 28-S ribosomal RNA migrates. A similar result was obtained in Northern blot analysis with SC-4, a cDNA probe which contains the 5′ half of the N-CAM cDNA (data not shown).

### 3.3. Isolation of Human SCLC N-CAM cDNAs

For isolation of cDNA clones encoding N-CAM in SCLC, we constructed a λgt10 cDNA library from poly(A)$^+$ RNA of a SCLC cell line, OS2-R. Approximately $5 \times 10^5$ phages of OS2-R cDNA library were screened by plaque hybridization with cDNA N-CAM probe, N294. Six positive plaques were obtained and plaque purified. The largest cDNA clone designated SC-2 consisted of two *Eco*RI fragments of 1.4 and 1.3 kbp. These fragments were subcloned into plasmid, and then subjected to partial DNA sequence analysis. Consequently, this clone lacked approximately 70 base pairs of N-terminal and signal peptides coding region. One of the other clones, SC-4, was also subcloned and partially sequenced. This clone had 1.7-kbp *Eco*RI fragment which contains the 5′ half of the N-CAM cDNA. These clones were aligned as shown in Fig. 3.

### 3.4. Sequence Analysis of Clones SC-2 and SC-4

Suitable DNA fragments of SC-2 and SC-4 were subcloned and sequenced. These clones consisted of 2960 nucleotides comprising a single open reading frame encoding 829 amino acids, and 176 bp of 5′ end and 190 bp of 3′ end of the cDNA are untranslated sequence (Fig. 4). By analogy with other N-CAMs, the first ATG sequence represents the start site of translation and the subsequent 57 bp encode a 19-amino acid hydrophobic signal peptides which result in leucine being $NH_2$-

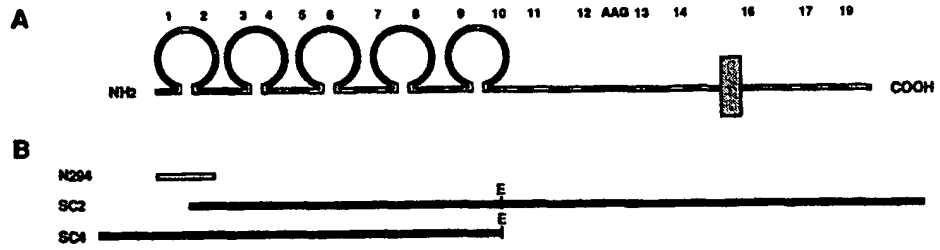


Fig. 3. Schematic representation of N-CAM cDNA clones and its predicted protein. (A) Domain structure of the human N-CAM expressed in SCLC. The exon numbers found in this isoform are listed above, and the position of each boundary is indicated by a transverse line. The five extracellular immunoglobulin domains are represented by loops, the cell membrane by a hatched vertical bar (Owens et al., 1987), and mini exon AAG located between exons 12 and 13 by a solid bar. (B) Porbe N294 used Northern blotting and cDNA library screening is indicated as a horizontal open bar, and cDNA clones SC2 and SC4 as horizontal solid bars under the corresponding positions of the schema. An internal *Eco*RI (E) site is indicated by a vertical line.

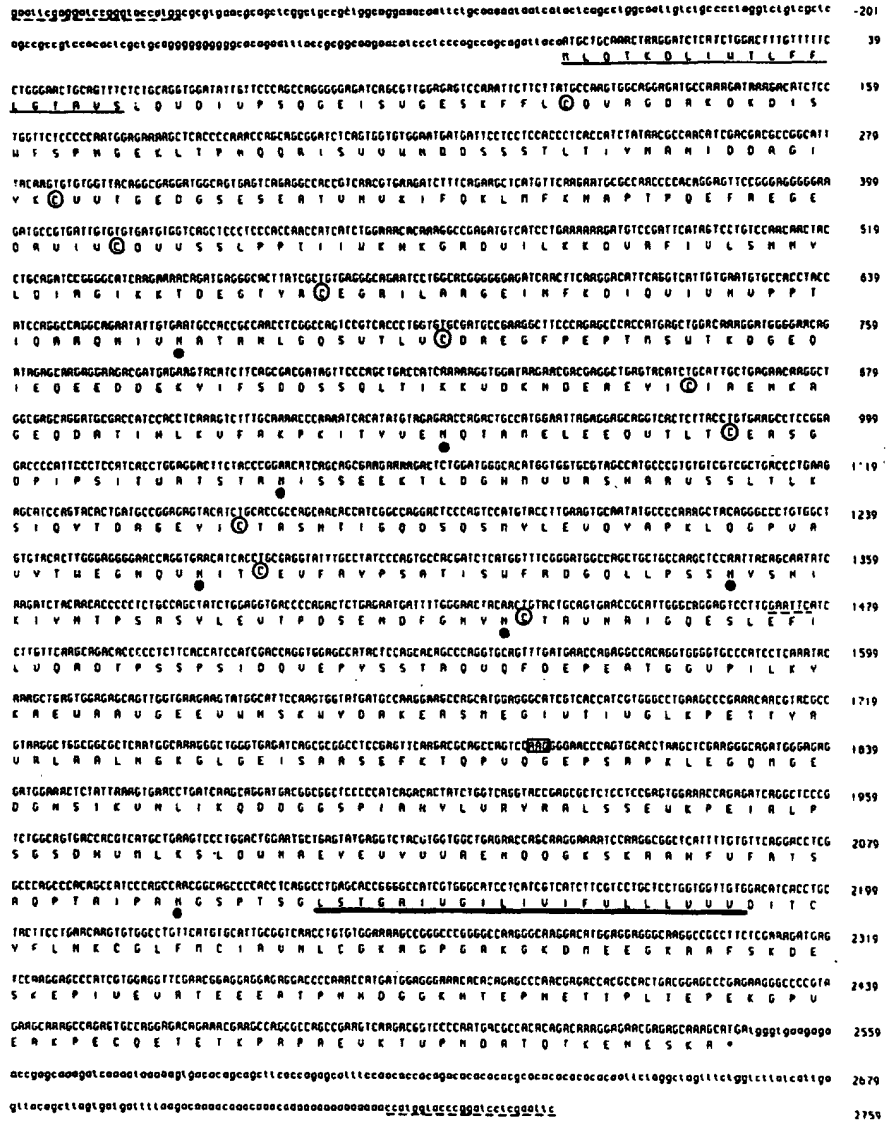

Fig. 4. Nucleotide and predicted amino acid sequence of the full length N-CAM-coding region for the 145-kDa isoform from a human SCLC cell line OS2-R. Nucleotides are numbered on the right from the initiation ATG codon and deduced amino acid are on the left. The presumptive signal peptide is underlined, and the membrane-spanning segment is indicated by a heavy bar. The ten cystein residues which make five intramolecular disulphide bridges are circled and seven consensus sites for N-linked glycosylation within the central region of the derived protein sequence are shown as black dots. Synthetic oligonucleotide adaptors containing sites for the restriction endonuclease *Eco*RI as well as an internal natural *Eco*RI site are indicated by dotted underlines. The mini exon AAG is enclosed in box.

terminal in the mature polypeptides. The extracellular region of the molecule contains seven putative N-glycosylation sites. Following 21 amino acids transmembrane segment, small cytoplasmic domain is present, lacking exon 18. In addition to usual exons, mini exon AAG triplet is found between exons 12 and 13. This SCLC- derived N-CAM cDNA is identical to N-CAM cDNA isolated from a human neuroblastoma cell line [19], with exceptions of lacking the VASE exon and of substitution G to T at position 1620 without changing encoding amino acid.

## 4. Discussion

This study indicates that the 145-kDa protein coded by 6.2-kb mRNA is the major isoform of N-CAM expressed in SCLC cell lines. Carbone et al. have reported a similar finding that N-CAM positive SCLC and NSCLC cell lines demonstrate a single 6.2-kb mRNA, using the cDNA clone encoding NKH-1 antigen as the probe in the Northern blot analysis [21]. Their report, however, demonstrating the correlation between the mRNA splicing patterns and the neuroendocrine phenotype, lacks any information available for comparing differences in the primary structure of the isoform between neuroendocrine cells and SCLC. Such information may be obtained by sequencing the full length cDNA encoding the 145-kDa N-CAM isoform of SCLC.

Alternative splicing forms of N-CAM expressed in SCLC have been reported with different message sizes, sometimes multiple forms in a single cell type. At the protein level, the N-CAM isoform reported varies from 95 kDa to 180 kDa [20,22,36,37]. Similarly, other size classes of N-CAM mRNA than the 6.2-kb mRNA have been demonstrated, although the transcripts are faint in general [20,22]. Furthermore, repeating PCR of N-CAM cDNA has revealed trace amounts of MSD1 product in an SCLC cell line [21]. These results indicate that many N-CAM isoforms may be detected by using highly sensitive methods, although the role of the isoforms at the small amount is not clear. Collectively, these results indicate that the low sialylated 145-kDa N-CAM translated from 6.2-kb mRNA is the most fundamental isoform in SCLC, while some SCLC cell lines may express 180-kDa N-CAM isoform translated from 7.4-kb mRNA.

To determine the cDNA sequence of SCLC-derived N-CAM, we selected several cDNA clones encoding N-CAM isolated from OS2-R, an SCLC cell line established in our laboratory. Based on the analysis of cDNA obtained from two clones, the cDNA sequence was shown to be essentially identical to that encoding 140-kDa N-CAM isoform of a human neuroblastoma cell line [19]. This finding confirms the possibility that SCLC is a representative tumor associating with neuroendocrine differentiation.

In addition to exons 1–14, 16, 17 and 19, triplet AAG, which was alternatively spliced with or without other alternatively spliced exons (SEC, MSD1), is found as an insert between exons 12 and 13. The MSD1 is a site of post-translational modification with O-linked carbohydrate, which is characteristic to hinge regions in other immunogloblin gene superfamilies. This triplet AAG is found independently in normal brain [17], muscle [38], NK cell [16], neuroblastoma [19], and SCLC cell line

H69 [22]. This subtle change in amino acid structure may represent an important point for modulation of cell-cell interaction in normal tissue, e.g. skeletal muscle, but it remains unclear in this tumor.

There are two differences in the cDNA sequence encoding the N-CAM isoform between OS2-R and the neuroblastoma line [19]. One is that OS2-R lacks a 30-bp VASE exon inserted between exons 7 and 8. The exon, causing a change in the predicted loop structure from an immunoglobulin constant region-like domain to a variable region-like domain, has been suggested to play a role in the modulation of synaptic plasticity including the neurite outgrowth inhibition. For example, whereas ~3% of N-CAM transcripts have VASE during early brain development, this progressively increases to ~50% of all transcripts in the adult central nervous system [39]. In SCLC, Van Duijnhoven et al. detected the exon in all N-CAM mRNA obtained from several cell lines [20]. Similarly, Moolenaar et al. demonstrated it in all size classes of N-CAM mRNA obtained from H69 cells [22]. These results confirm the findings that all N-CAM isoforms consist of two forms, with or without VASE exon at various proportion [18,40]. Therefore, it seems likely that the lacking of VASE in the sequenced cDNAs, SC-2 and SC-4, does not exclude the possibility of presence of VASE in mRNA of OS2-R cells. The other is not a significant difference, because it is a single base pair change G to T at position 1620 which results in no change in the encoded amino acid.

Finally, SCLC-derived N-CAM cDNA did not contain any portion specific for the histological type but was essentially identical to that encoding neuroblastoma, indicating that no therapeutic approach specific for SCLC would be provided from the characteristics of SCLC-derived N-CAM.

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